L-Arginine counteracts nitric oxide deficiency and improves the recovery phase of ischemic acute renal failure in rats

Reinhard Schneider, Ulrike Raff, Nicole Vornberger, Monika Schmidt, Ralf Freund, Mark Reber, Lothar Schramm, Stepan Gambaryan, Christoph Wanner, Harald H.H.W. Schmidt, and Jan Galle

Division of Nephrology, Department of Medicine, Julius-Maximilians-University, Würzburg, Germany; and Rudolf-Buchheim-Institute for Pharmacology, Justus-Liebig-University, Gießen, Germany

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**Background.** In ischemic acute renal failure (ARF), nitric oxide–dependent regulation of renal hemodynamics and glomerular function is disturbed. Previous studies indicate that the nitric oxide precursor L-arginine (L-Arg) has beneficial effects on renal function. Here we further analyzed the impact of L-Arg on functional and biochemical parameters of nitric oxide signaling during the course of ischemic ARF.

**Methods.** Ischemic ARF was induced in rats by bilateral clamping of renal arteries for 45 minutes. L-Arg was applied intraperitoneally during clamping, and orally during 14 days of follow-up. Glomerular filtration rate (GFR) and renal plasma flow (RPF) were measured, and biochemical parameters analyzed by protein immunoblots.

**Results.** Clamping resulted in 70% to 90% reduction of GFR and RPF, with a gradual recovery by day 14. Using an in situ assay with the oxidative fluorescent dye hydroethidine, increased tubular generation of O$_2^-$ was detected in the early course of ischemic ARF, indicating enhanced oxidative stress. These findings were accompanied by up-regulation of the nitric oxide receptor, soluble guanylate cyclase, and by significant regulatory changes of inducible nitric oxide synthase (iNOS) and endothelial NOS expression. L-Arg had a beneficial effect on GFR and RPF, decreased O$_2^-$ production, diminished up- or activating cGMP-dependent protein kinases (cGK-I and cGK-II) [15].

**Conclusion.** Ischemic ARF is accompanied by marked alterations in the expression of key enzymes of the nitric oxide pathway, indicative for deficiency of constitutive NOS activity. L-Arg supplementation reduces O$_2^-$ generation and significantly improves the expression of nitric oxide signaling proteins as well as the recovery phase of ischemic ARF.

Acute renal failure (ARF) is a common clinical comp-

Key words: renal failure, L-arginine, ischemia/reperfusion injury, hydroethidine, nitric oxide, superoxide.

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failure [24], but only limited information is available on prolonged effects of \( \text{L-Arg} \) on ischemic ARF and on the downstream cascade of nitric oxide. Therefore, we analyzed the impact of \( \text{L-Arg} \) supplementation on kidney function during the recovery phase of ischemic ARF over 14 days. For this purpose, we induced renal ischemia in a rat model by bilateral clamping of renal arteries, followed by reperfusion. Supplementation of \( \text{L-Arg} \) was started within the phase of ischemia and was continued throughout the follow-up period of 14 days. Kidney function was monitored by measuring glomerular filtration rate (GFR) and renal plasma flow (RPF). The renal expression of NOS, nitric oxide receptor soluble guanylyl cyclase, cGMP-dependent protein kinase (cGK-I), and \( \text{O}_2 \) generation visualized with hydroethidine fluorescence using confocal laser scan microscopy were determined.

METHODS

Drugs

\( \text{L-Arg} \), \( \text{D-arginine (D-Arg)} \), \( \text{L-aspartic acid (L-Asp)} \), \( \text{L-arginine L-aspartic acid (L-Arg)} \), \( \text{D-arginine L-aspartic acid (D-Arg)} \) paraformaldehyde, and hydroethidine were from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescence-inulin and paraminohippuric acid (PAH) were from ICN Pharmaceuticals, Inc. (Costa Mesa, CA, USA), tramadol (Tramal®) was from Grünenthal (Aachen, Germany), xylacin hydrochloride (Rompun®) was from Bayer (Leverkusen, Germany), and ketamine (Ketanest®) was from Pharmacia & Upjohn (Erlangen, Germany). If not indicated otherwise, all drugs were further diluted in 0.9% NaCl (wt/vol).

Experimental procedure

Female Sprague-Dawley rats (200 to 250 g body weight) were obtained from Charles River (Kissleg, Germany). After a period of at least 24 hours in cages within a temperature-controlled room with 12–12-hour light-dark cycle, standard food with free access to drinking water, anesthesia was performed by intraperitoneal application of xylacin hydrochloride (10 mg/kg body weight) and ketamine (100 mg/kg body weight). All operative procedures were performed on thermoregulated heating boards to maintain body temperature at 37.5°C. Animals were divided into the following subgroups.

Clamping group (bilateral clamping and supplementation with saline). Both kidneys were prepared carefully by a bilateral flank incision. Renal arteries were prepared and temporarily ligated on both sides in order to start clamping with microclips simultaneously. Dispensable fluid loss was prevented by closing wounds with sterile strips during the clamping phase. Intraportal application of 0.5 mL 0.9% NaCl was started 15 minutes after clamping. Following the clamping period, both microclips were removed and sutures of muscular layers and skin were made to close the wound. For postoperative prophylaxis of pain, tramadol (2.0 mg/kg body weight) was applied subcutaneously.

\( \text{L-Arg} \) group (bilateral clamping and supplementation with \( \text{L-Arg} \)). Identical procedure was performed as described for clamping group, except interperitoneal supplementation of \( \text{L-Arg} \) (500 mg/kg body weight) in 0.5 mL 0.9% NaCl after 15 minutes of arterial clamping. During the follow-up period, \( \text{L-Arg} \) was added to tap water (1%) [25].

To control for effects resulting from protein supplementation, the stereoisomeric \( \text{D-Arg} \) was used in the same manner as \( \text{L-Arg} \). In order to apply comparable concentrations of \( \text{D-Arg} \), preliminary dose finding experiments analyzing the pharmacokinetics of oral and intraperitoneal supplementation of \( \text{L-Arg} \) and \( \text{D-Arg} \) were performed as described below.

Sham group (sham-operation and supplementation with saline). Identical procedure was performed as described for clamping group, except that in these animals no clamping of renal arteries was performed. A dose of 0.9% NaCl (0.5 mL) were applied intraperitoneally after 15 minutes of arterial clamping.

Control group (untreated animals). Animals with no previous treatment performed were investigated. These animals reflect day 0.

The care of animals and experimental procedures performed in this study were in accordance with the German law for animal protection.

Pharmacokinetics and effects on blood pressure

Left femoral artery and vein of anesthetized rats were prepared. Arterial blood pressure was measured using a pressure transducer (Hellige Recomed, Freiburg i. Br., Germany), which was placed in the cannulated femoral artery. Values of mean arterial pressure (MAP) and heart rate were recorded continuously for 6 hours to exclude hemodynamic effects of the \( \text{L-Arg} \) application on MAP or heart rate. Blood samples were taken as control prior to application of any substance, and at 6 time points thereafter. Enteral application of \( \text{L-Arg} \) and \( \text{D-Arg} \) was performed via direct gastric infusion after laparotomy. Fluid loss was prevented by immediate closure of wounds. All blood samples were commercially analyzed by Bioscientia (Ingelheim, Germany) with high-performance liquid chromatography (HPLC) analysis for serum concentrations of \( \text{L-Arg} \) and \( \text{D-Arg} \) in order to calculate pharmacokinetics.

Measurement of inulin and PAH clearance

For estimation of GFR and RPF, inulin and PAH clearances were determined. The left femoral vein was cannulated with a PE-10 (polyethylene) catheter. Inulin and PAH (1 mg of each substance solved in 0.25 mL 0.9% NaCl) were applied as a bolus injection, followed by constant infusion of both substances (inulin 5 mg/hour,
PAH 5 mg/hour) using a Secura FT perfusor (B. Braun, Melsungen, Germany). After suprapubic incision, the urine bladder was cannulated with a PE-50 (polyethylene) catheter to measure urine flow and obtain urine samples. When reaching a steady state after 30 minutes of infusion, urine was collected for 20 minutes and blood samples were drawn subsequently. Samples were centrifuged and stored at −20°C. Inulin concentrations in urine and plasma were determined by fluorescence spectrometry. PAH concentrations were measured by photospectrometry (Dynatech Lab, Guernsey, UK) using the antherone method. Calculations of GFR and RPF were performed according to the equations:

\[ \text{GFR} = \frac{(I_U \cdot V_U)}{(I_P \cdot t)} \]

and

\[ \text{RPF} = \frac{(U_{PAH} \cdot V_U)}{(P_{PAH} \cdot t)} \]

where \( I_U \) is inulin concentration in urine; \( U_{PAH} \) is PAH concentration in urine; \( I_P \) is inulin concentration in plasma; \( P_{PAH} \) is PAH concentration in plasma; \( V_U \) is urine volume; and \( t \) is time of measurement.

Organ preparation and tissue harvesting

After drawing blood samples, both kidneys were perfused under pressure-controlled conditions (100 mm Hg) with either ice-cold 0.9% NaCl for 20 seconds for Western blot analysis, or paraformaldehyde 4% in phosphate-buffered saline (PBS) for hydroethidine fluorescence confocal laser microscopy. Kidneys were removed, and for some experiments, dissected into cortex, medulla (outer and inner stripe of the outer medulla), and papilla (inner medulla). Then, kidney zones and whole kidneys were snap-frozen in liquid nitrogen and stored at −80°C.

Protein immunoblot

For Western blot analysis, frozen whole kidneys were homogenized using a stainless steel mortar cooled by liquid nitrogen, dissolved in lysis buffer containing 25 mmol/L Tris-HCl, 7 mmol/L reduced glutathione, 0.5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2 mol/L phenylmethylsulfonyl fluoride (PMSF), 1 μmol/L leupeptin, 1 μmol/L pepstatin, 1 μmol/L trans-epoxysuccinyl-L-leucylamido butane, and 1 mg/mL trypsin inhibitor, and further minced with an ultrasonic disperser UW 70 (Bandelin Electronic, Berlin, Germany). For analysis of expression of cGMP-dependent protein kinase, different kidney zones, dissected into cortex and medulla, were used. Total protein was measured in samples using the Bradford method [26]. Samples of protein (5 to 40 μg) were analyzed by Western blot with the respective antibodies. Rabbit iNOS polyclonal antibody (diluted 1:1000) and mouse eNOS monoclonal antibody (diluted 1:250) were from Transduction Laboratories (Lexington, KY, USA); cGK-I polyclonal antibody (diluted 1:200), rabbit soluble guanylyl cyclase subunit α (diluted 1:1000) and soluble guanylyl cyclase subunit β (diluted 1:1000) polyclonal antibodies were a kind gift of Dr. Zabel [11]. Blots were subsequently incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Dako, Hamburg, Germany) and were developed using a chemiluminescence kit (ECL Plus) following the manufacturer’s instruction (Amersham Pharmacia Biotech, Buckinghamshire, UK). Blots were analyzed densitometrically using the Quantity One software (Bio-Rad Laboratories, Philadelphia, PA, USA). To describe variation of expression changes in the different blots, values were expressed in percentages of the respective sham group as defined in the legend of each figure.

Oxidative fluorescent microscopy

The oxidative fluorescent dye hydroethidine was used to evaluate in vitro production of superoxide (O\(_2^−\)) [27]. Hydroethidine is oxidized to ethidiumbromide (EtBr) selectively by O\(_2^−\) and is freely permeable to cells where it is trapped by intercalating with the DNA [28]. When oxidized, EtBr is excited at 488 nm with an emission spectrum of 610 nm.

Animals, anesthetized as described before, received 50 μg/100 g body weight hydrothidine (diluted in 10% dimethylsulfoxide in PBS 500 μL/100 g body weight) intravenously [injected into the cannulated femoral vein for 30 minutes using a Secura FT perfusor (B. Braun)]. After 30 minutes of equilibration, animals were sacrificed. Tissue was harvested and organs were fixed as described before, and shock-frozen in liquid nitrogen. Cryoprotected frozen kidney sections (5 μm) were prepared and immediately scanned using a MRC-1024 laser scanning confocal microscope (Bio-Rad Laboratories) equipped with a krypton/argon laser and an Axiovert 135 TV microscope (Carl Zeiss, Inc., Oberkochen, Germany). Fluorescence was detected with a 585 nm long-pass filter. Laser settings were identical for acquisition of images from day 1, 3, 7, and 14 of l-Arg and clamping group as well as specimens of sham group. For semiquantitative evaluation of superoxide formation, four to six images of each treatment group were analyzed densitometrically using the Quantity One software (Bio-Rad Laboratories). To describe variation of fluorescence intensity, values were expressed as the percentage of the respective sham group.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis between experimental groups was performed by applying the t test for unpaired groups. In Figures 1, 2B, 3, 4, 5, and 6, “sham” was set to 100%, and data of the treatment groups “clamping” and “clamping + l-Arg” are expressed as variation from “sham.” The N value refers to the number of animals used in the experiments. A probability value \( P < 0.05 \) was considered statistically significant.
rate was below 5% in all treatment groups (data not shown).

GFR and RPF in the course of ischemic ARF

Inulin clearance was measured to calculate GFR, and PAH clearance to estimate RPF. In the clamping group, there was a strong decline in inulin (Fig. 1A) and PAH clearance (Fig. 1B) when compared to sham group, with a minimum on day 1 of the follow-up period after ischemic ARF (inulin clearance, $0.28 \pm 0.11 \text{ mL/min/100 g body weight}$ in clamping group vs. $0.8 \pm 0.25 \text{ mL/min/100 g body weight}$ in sham group; PAH clearance, $0.15 \pm 0.13 \text{ mL/min/100 g body weight}$ in clamping group vs. $1.88 \pm 0.55 \text{ mL/min/100 g body weight}$ in sham group). During the following days, GFR and RPF improved in a nearly linear manner to reach 73.5% of inulin clearance of sham group on day 14, and 58.0% of PAH clearance of sham group on day 11 (Fig. 1). In L-Arg–substituted animals, inulin and PAH clearances were also lowest on day 1 after induction of ischemic ARF, but significantly improved during the 14 days of follow-up when compared to clamping group (Fig. 1). In contrast, application of the stereoisomeric d-Arg had no influence on inulin and PAH clearances compared to clamping group (data not shown).

Superoxide radical detection

Impaired renal function during the course of ischemic ARF could partly result from increased oxidative stress. We therefore attempted to directly measure oxygen radical formation in renal tissue. By use of confocal microscopy, kidney sections of clamping group demonstrated a marked increase in cortical EtBr fluorescence, with the highest intensity at the first day after intervention and a gradual decrease during follow-up, reflecting an increase in O$_2^-$. L-Arg supplementation lowered EtBr fluorescence at all time points. EtBr fluorescence was lowest in sham-treated animals (Fig. 2 A). A semiquantitative evaluation of superoxide formation is presented in Figure 2 B. Combining histologic and fluorescence pictures of animals evaluated on day 1 revealed that fluorescence intensity was highest in tubular structures, while it was less pronounced in glomerula or interstitial matrix (Fig. 2 C). EtBr fluorescence was distinctly stronger in cortical kidney segments compared to medullary segments in all groups analyzed (data not shown).

Expression of NOS

Expression of NOS, particularly the balance of iNOS and eNOS, are considered to play a major role in the course of ischemic ARF. Evaluating exemplary days during follow-up after initiation of ischemic ARF, we detected a fourfold and, compared to sham, significant up-regulation of iNOS, peaking on day 1 (Fig. 3). Expression of iNOS in L-Arg–treated animals was also stronger com-
Table 1. Arginine serum concentrations after intraperitoneal and oral application of L-arginine (L-Arg) and D-arginine (D-Arg)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Treatment dose</th>
<th>Control</th>
<th>30 minutes</th>
<th>60 minutes</th>
<th>120 minutes</th>
<th>240 minutes</th>
<th>360 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arg</td>
<td>500 mg/kg</td>
<td>2.8 ± 1.9</td>
<td>21.3 ± 15</td>
<td>12.5 ± 8.8</td>
<td>5.3 ± 3.7</td>
<td>2.5 ± 1.8</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td>D-Arg</td>
<td>500 mg/kg</td>
<td>1.8 ± 1.2</td>
<td>44.5 ± 31</td>
<td>31.0 ± 21</td>
<td>21.3 ± 15</td>
<td>9.0 ± 6.4</td>
<td>5.8 ± 4.1</td>
</tr>
<tr>
<td>D-Arg</td>
<td>125 mg/kg</td>
<td>2.3 ± 1.6</td>
<td>14.8 ± 10</td>
<td>14.8 ± 10</td>
<td>11.5 ± 8.1</td>
<td>6.3 ± 4.4</td>
<td>3.3 ± 2.3</td>
</tr>
<tr>
<td>Oral</td>
<td>L-Arg 500 mg/kg</td>
<td>2.0 ± 1.4</td>
<td>4.8 ± 3.4</td>
<td>2.5 ± 1.8</td>
<td>2.0 ± 1.4</td>
<td>1.5 ± 1.1</td>
<td>1.0 ± 0.7</td>
</tr>
<tr>
<td>Oral</td>
<td>D-Arg 500 mg/kg</td>
<td>2.3 ± 1.6</td>
<td>10.5 ± 7.4</td>
<td>14.0 ± 9.9</td>
<td>15.3 ± 11</td>
<td>12.0 ± 8.5</td>
<td>8.8 ± 6.2</td>
</tr>
<tr>
<td>Oral</td>
<td>D-Arg 62.5 mg/kg</td>
<td>2.5 ± 1.8</td>
<td>3.3 ± 2.3</td>
<td>3.3 ± 2.3</td>
<td>3.3 ± 2.3</td>
<td>3.0 ± 1.2</td>
<td>3.5 ± 2.5</td>
</tr>
</tbody>
</table>

Fig. 2. Influence of L-arginine (L-Arg) supplementation on superoxide radical generation visualized by ethidium bromide (EtBr) fluorescence. (A) Representative confocal laser microscopy (magnification 100 × 1) fluorescence scanning images of cortical kidney specimen of sham (S), clamping (C), and clamping + L-Arg group (C + LA) (arranged in lines) on days 1, 3, 7, and 14 after ischemic acute renal failure (ARF). (B) Semiquantitative evaluation of superoxide formation by densitometrical detection of fluorescence intensity on days 1, 3, 7, and 14 after ischemic ARF. (C) Representative images of the histologic tissue structure (a), fluorescence image (b), and an overlay picture of both (c) on the first day of follow up after ischemic ARF for sham (S), clamping (C), and clamping + L-Arg and group (C + LA) (arranged in lines; magnification 100 × 1).
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Fig. 3. Impact of l-arginine (l-Arg) supplementation on inducible nitric oxide synthase (iNOS) expression in ischemic acute renal failure (ARF). (A) Representative Western blot analysis of iNOS expression in whole kidney homogenates of clamping group (top line) and l-Arg group (bottom line) at the time points 1, 3, 7, and 14 days after ischemic ARF. (B) Effect of l-Arg supplementation on iNOS expression compared to clamping group during 14 days of follow-up after ischemic ARF. Data of the treatment groups “clamping” and “clamping + l-arginine” are expressed as variation from “sham.” *P < 0.05 clamping group vs. l-Arg group, N = 3 animals for each individual time point.

Fig. 4. Impact of l-arginine (l-Arg) supplementation on the expression of endothelial nitric oxide synthase (eNOS). (A) Representative Western blot analysis of eNOS expression in whole kidney homogenates of clamping group (top line) and l-Arg group (bottom line) at the time points 1, 3, 7, 9, and 14 days after ischemic ARF. (B) Effect of l-Arg supplementation on eNOS expression compared to clamping group during 14 days of follow-up after ischemic ARF. Data of the treatment groups “clamping” and “clamping + l-arginine” are expressed as variation from “sham.” *P < 0.05 clamping group vs. l-Arg group, N = 5 to 8 animals for each individual time point.

pared to sham, albeit the increase in expression was only 1.6-fold.

eNOS was slightly up-regulated on the first days after intervention (Fig. 4) with a significantly decreased eNOS expression in l-Arg group when compared to clamping group on the second day of follow-up. In the second week of postinterventional follow-up, there was a trend for down-regulation of eNOS, albeit this did not reach statistical significance (0.05 < P < 0.1) (Fig. 4). A representative Western blot analysis of eNOS expression in both treatment groups clamping and l-Arg is depicted in Figure 4.

Expression of soluble guanylyl cyclase after ischemic ARF

Increased O$_2^-$ formation, together with an imbalance in the expression of iNOS and eNOS, might lead to a functional nitric oxide deficiency in ischemic ARF [29]. In order to evaluate this hypothesis, we measured expression of the nitric oxide receptor soluble guanylyl cyclase. Ischemic ARF resulted in an upregulation of soluble guanylyl cyclase β expression, with a maximal expression on day 11 in the clamping group (Fig. 5). l-Arg treatment significantly prevented upregulation of soluble guanylyl cyclase. Representative Western blots of soluble guanylyl cyclase expression during the follow-up period are shown in Figure 5. Similar changes as in the expression of soluble guanylyl cyclase β were detected for soluble guanylyl cyclase α (data not shown).

Expression of cGK-I

In order to analyze whether soluble guanylyl cyclase up-regulation affected the signaling cascade downstream of the nitric oxide/cGMP pathway, we measured the expression of cGMP-dependent protein kinases, cGK-I and cGK-II. Cortical and medullary expression of cGK-I, as well as expression of cGK-II in the clamping group, did not differ from sham group and was not influenced by l-Arg treatment during the follow-up after ischemic ARF (representative blots of cortical cGK-I expression are depicted in Fig. 6).

DISCUSSION

We established an animal model of ischemic ARF and closely monitored several parameters of renal function over a prolonged follow-up period of 14 days. Furthermore, we analyzed potential biochemical mechanisms involved in renal ischemia/reperfusion (I/R) injury. Particular emphasis was placed on the nitric oxide pathway and on oxidative stress. Finally, the impact of l-Arg supplementation on renal function and biochemical markers of nitric oxide signaling was investigated.

Our model of ischemic ARF resulted in a significant and, over 14 days, partly reversible reduction of GFR.
and RPF. Laser scan microscopy using the fluorescent dye hydroethidine indicated enhanced in situ $O_2^·$ formation, predominantly in the renal cortex. Enhanced $O_2^·$ formation was indirectly confirmed by enhanced expression of nitrotyrosine. This was associated with an early up-regulation of iNOS, a late moderate decrease in eNOS expression, and a continuous rise in expression of the nitric oxide receptor soluble guanylyl cyclase, suggestive for substantial nitric oxide deficiency. Expression of cGK-I was not altered. l-Arg supplementation improved the recovery phase of renal failure, lowered cortical $O_2^·$ formation, prevented almost completely up-regulation of soluble guanylyl cyclase and of iNOS, and prevented partly up-regulation of NT.

These results further corroborate and extend that the nitric oxide pathway is fundamentally altered in ARF [4, 5]. Previous studies examined short-term I/R injury; our present experimental design allowed observations over a prolonged period of time. Here we show that alterations of the nitric oxide pathway take place not only during the acute phase of ARF, but rather last until the end of the recovery phase. In particular, our findings indicate that it is important to differentiate between initial changes and resulting consequences for the recovery phase. Up-regulation of iNOS is an early event that may lead temporarily to increased levels of nitric oxide. In contrast to eNOS-derived nitric oxide, nitric oxide produced by the iNOS seems to be causally linked to the course of ARF [30], and the balance of iNOS and eNOS plays an important role [29]. In particular in the setting of concomitant oxidative stress [29], as it was the case in our experiments, nitric oxide derived from iNOS up-regulation will react with $O_2^·$, and significant amounts of peroxynitrite (ONOO⁻) will be formed [31], leading to nitrosative stress [29]. Indeed, iNOS-dependent NT formation has been shown in other I/R models (e.g., of the brain [32], and enhanced NT formation has been found frequently in ARF [30, 33, 34]).

Our general interpretation is that renal ischemia leads to decreased nitric oxide bioavailability, despite initial up-regulation of iNOS. This assumption is based primarily on the up-regulation of the nitric oxide receptor soluble guanylyl cyclase during the recovery phase of ischemic ARF, together with the moderate down-regulation of eNOS and the beneficial effect of l-Arg. In this context, one has to distinguish between nitric oxide derived from constitutive eNOS and from the iNOS. While eNOS-derived nitric oxide is believed to be responsible for maintaining physiologic renal hemodynamics and functions, iNOS-derived nitric oxide predominantly elicits pathologic effects [35]. Thus, even under the condition of up-regulated iNOS, there may be a relative nitric oxide deficiency at sites of physiologic nitric oxide action.

It is commonly accepted that in states of nitric oxide deficiency soluble guanylyl cyclase sensitivity is up-regu-
lated [36, 37]. Decreased nitric oxide activity in the course of ARF has been described in various short-term experimental models [22, 38, 39]. Our observation of a potent up-regulation of soluble guanylyl cyclase in the follow-up period emphasizes the importance of a relative nitric oxide deficiency in ischemic ARF.

Nitric oxide deficiency may result from decreased formation or increased degradation. The results of our present study provide evidence for both mechanisms. It has been suggested that the balance between expression and activity of the inducible and constitutive isoforms of NOS is disturbed in ARF, and that these changes contribute to ARF pathophysiology [35]. In particular, up-regulation of iNOS may lead to down-regulation of eNOS [40]. Our data showing initial up-regulation of iNOS shortly after induction of ischemic ARF confirm previous reports [34, 41]. The moderate down-regulation of eNOS may be a consequence of iNOS up-regulation and is well in line with the hypothesis of an imbalance between eNOS and iNOS in ischemic ARF.

However, increased inactivation of nitric oxide may also be relevant for our observations. As outlined above, nitric oxide rapidly reacts with O$_2^-$, resulting in the formation of the highly reactive oxidant ONOO$^- [31]$ and scavenging of nitric oxide [42]. ONOO$^-$ itself is capable of causing lipid peroxidation and DNA damage. O$_2^-$ represents an important member of the reactive oxygen species (ROS) family, and its formation during I/R injury has been demonstrated frequently [43]. Potential sources for enhanced O$_2^-$ formation in the kidney are nicotinamide adenine dinucleotide phosphate (NADPH) oxido- dases (Nox) [44], xanthine oxidase (XO) [45] or, in case of l-Arg deficiency, uncoupled NOS itself [46]. Another factor that might contribute to enhanced oxidative stress in ischemic ARF has been reported in experimentally induced renal warm ischemia. In this rat model, enzymatic activity of various antioxidants [superoxide dismutase (SOD), glutathion peroxidase, catalase] was significantly reduced [47].

The beneficial effect of the nitric oxide precursor l-Arg on the course of ischemic ARF provides the third line of evidence for a functional nitric oxide deficiency. l-Arg not only improved GFR and RPF, but prevented almost completely the up-regulation of soluble guanylyl cyclase and of iNOS, partially the up-regulation of NT, and reduced O$_2^-$ formation. This is suggestive for a relative l-Arg deficiency during I/R injury, as it has been described before [22, 48]. In addition to beneficial effects of l-Arg on renal hemodynamics, Jerkic et al [48] observed a cytotoxic protective influence of l-Arg after induction of ischemic ARF. However, it should be noted that the impact of l-Arg on cell survival under the condition of hypoxia/reoxygenation is equivocal. While acute l-Arg was beneficial for the course of renal ischemia in an in vivo rat model, it worsened in vitro cellular injury of proximal tubular cells in terms of lactate dehydrogenase (LDH) release after hypoxia/reoxygenation [49], and increased lipid peroxidation after l-Arg infusion has also been observed [50]. In the study by Schramm et al [22], using a model of toxic ARF, tissue l-Arg levels were reduced, but still clearly above the $K_v$ value for saturation of NOS [22]. However, supplementation of l-Arg had beneficial effects for kidney function, describing the so-called “l-Arg paradox” [23]. Various mechanisms have been discussed for this perplexing effect of l-Arg [23]. Those include compartmentalization of the l-Arg pool in the cytoplasm, leading to lower concentrations in vicinity to the eNOS than expected from levels in the serum or tissue homogenates. A recent study by McDonald et al [51] indicates that eNOS is colocalized in cytoplasmic membrane caveolea with the cationic amino acid transporter 1 (CAT-1). As a result, extracellular l-Arg might be utilized rapidly by eNOS in such a micro-environment, and tissue or serum levels of l-Arg do not necessarily reflect the substrate pool available for the enzyme. This may be of particular relevance in the setting of ischemic ARF, since it has been shown recently that mRNA of CAT-2, another cationic amino acid transport- er, is up-regulated in ischemic ARF, leading to augmented l-Arg transport [52]. Alternatively, the NOS inhibitory effect of asymmetric dimethylarginine (ADMA), an endogenous molecule that accumulates in renal failure [53], could be overcome by excess l-Arg. Furthermore, relative l-Arg deficiency could result in an uncoupling of NOS, leading to O$_2^-$ formation instead of nitric oxide, as it has been described for tetrahydrobiopterin deficiency [54]. The fact that l-Arg lowered cortical O$_2^-$ formation in our model is well in line with this interpretation.

CONCLUSION

Ischemic ARF is accompanied by marked alterations in the expression of key enzymes of the nitric oxide pathway, indicative for deficiency of constitutive NOS activity. l-Arg supplementation reduces O$_2^-$ generation and significantly improves the expression of nitric oxide signaling proteins as well as the recovery phase of ischemic ARF.

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Reprint requests to Dr. Jan Galle, University Hospital, Department of Medicine, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany. E-mail: j.galle@mail.uni-wuerzburg.de
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